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Comments:

Attached are copies of Exhibits 1-5 submitted with the response filed May 11, 2004. Emily Tongco is out of the office on business until Thursday, so please call Brenda Campbell at (858) 720-7992 if you need anything further. Thanks very much.

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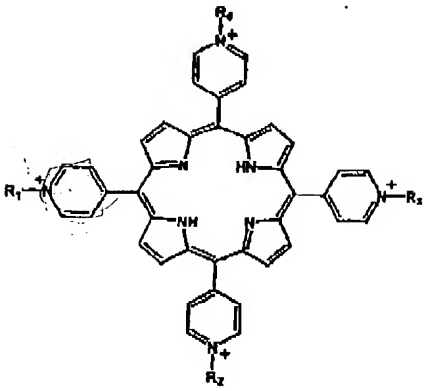
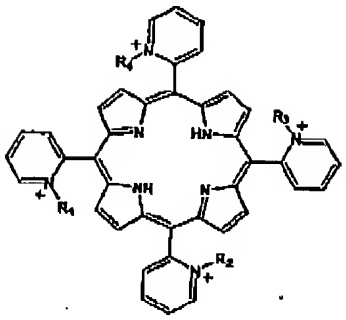




designations	R_1	R_2	R_3	R_4
5,10,15,20-tetrakis(*N*-methyl-4-pyridiniumyl)-21H,23H-porphyrin derivatives (left hand structure)				
1 TMPyP(4)	Me	Me	Me	Me
2 TBPyP(4)	Bu	Bu	Bu	Bu
3 TOPyP(4)	octyl	octyl	octyl	octyl
4	Me			
5	Me	Me		
6	Me		Me	
7	Me	Me	Me	
8	CH_2CH_2COOH			
9	$CH_2CH_2CH_2NH_2$			
10	$CH_2CH_2CH_2NH_2$	$CH_2CH_2CH_2NH_2$	$CH_2CH_2CH_2NH_2$	
5,10,15,20-tetrakis(*N*-methyl-2-pyridiniumyl)-21H,23H-porphyrin derivative (right hand structure)				
11 TMPyP(2)	Me	Me	Me	Me

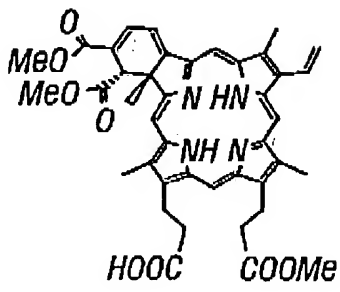
Fig. 1. Cationic porphyrins in this study.

I.D.] was built in a eCAP capillary cartridge (Beckman, 100×800 μm aperture). Phosphate buffer was prepared by dissolving NaH_2PO_4 or Na_2HPO_4 in deionized water and adjusting the solution to the desired pH with phosphoric acid or sodium hydroxide. All running solutions were filtered through a 0.2 μm-membrane filter (Gelman Science, FP-200) before use.

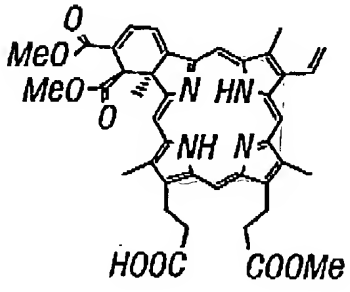
New capillary columns were rinsed with 0.1 *M* sodium hydroxide for 30 min, then with deionized water for 30 min, then with running buffer for 1 h. The capillary column was regenerated between runs with 0.1 *M* sodium hydroxide for 10–15 min, then with deionized water for 5–10 min, then with the running buffer for 10 min. A sample solution was prepared by dissolving a small amount of cationic porphyrin (1–2 mg) into deionized water (1 ml), then filtering it through a cotton or glass wool packed in a small pipet, then storing it in the dark. About 200 μl of sample solution was diluted by addition of about 50 μl of the running buffer to be used.

Separations were performed with normal polarity from the inlet vial (anode) to the outlet vial (cathode). Pressure injections of 3 s were used. Voltages were chosen in the range 10–20 kV, de-

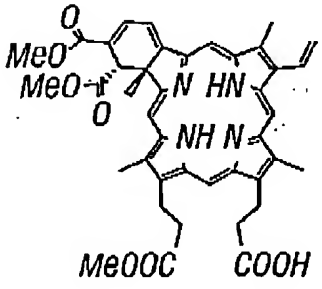
1/10



BPDMA (Ia-1)



BPDMA (Ia-2)



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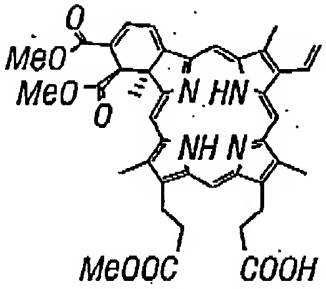

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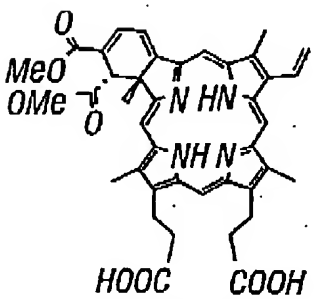
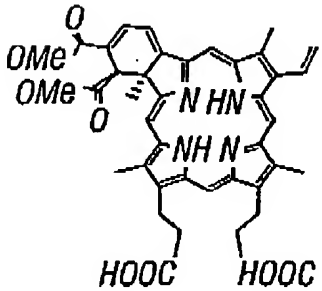


BPDDA (II-1)

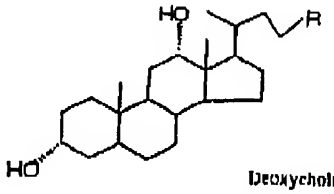


BPDDA (II-2)

FIG. 1

TABLE. I

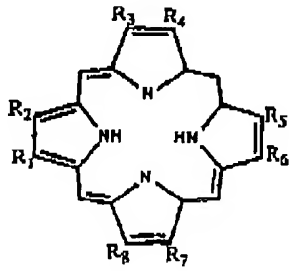
a) Structures of bile salts



Deoxycholate: R = COO^-

Taurodeoxycholate: R = $CONH(C_2H_4)SO_3^-$

b) Structures of Porphyrin



#	Porphyrin (isomer I)	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
1	Coproporphyrin	M	P	M	P	M	P	M	P
2	Pentaporphyrin	M	P	M	P	A	P	P	M
3	Mesoporphyrin	M	E	M	E	M	P	P	M
4	Hexaporphyrin	M	P	M	P	A	P	P	A
5	Heptaporphyrin	M	P	A	P	A	P	A	P
6	Uroporphyrin	A	P	A	P	A	P	A	P

Substituents: M = $-CH_3$; E = $-C_2H_5$; A = $-CH_2COOH$; P = $-CH_2CH_2COOH$.

Although this design is capable of optimizing several factors simultaneously, no fixed rules existed for the selection of the low and high levels and further experiments may have to be carried out based on conclusions from the optimized experimental conditions.

In our laboratory, we have developed several optimization schemes for CE separations, based on the overlapping resolution mapping (ORM) procedure [14]. In one study, two parameters, *i.e.*, buffer pH and β-cyclodextrin concentration, were optimized using a two-dimensional rectangular ORM scheme for the separation of eight sulphonamides [15]. In another investigation, the concentrations of α-, β- and γ-cyclodextrins were optimized for the CE separation of a group of plant growth hormones with a triangular ORM scheme [16]. To date, the use of a systematic optimization scheme for the CE separation of porphyrins has not been reported.

Naturally occurring porphyrins are intermediate metabolites of haem biosynthesis. Disturbances in the biosynthesis, caused by inborn or acquired defects of the corresponding enzymes, give rise to a family of diseases called porphyria. Depending on the break of the metabolic pathway, different intermediate porphyrins are subsequently formed and accumulated in body fluids and tissues. Porphyrins with different numbers of carboxylic acid groups have been conventionally separated by gradient reversed-phase high-performance liquid chromatography (HPLC) owing to their varying polarity [17]. While providing satisfactory results, the gradient HPLC procedures tended to be laborious and time consuming [17]. The ionizable carboxyl groups on these porphyrins, however, provide an advantage for separation in CE. Under appropriate conditions, they can give rise to different electrophoretic mobilities under an applied voltage [18]. Consequently, it was considered worthwhile to investigate systematic approaches which can be employed to obtain the optimum conditions for the separation of porphyrins by CE. In this investigation, the parameters chosen for the optimization study were sodium dodecyl sulphate (SDS) concentration, percentage of N,N-dimethylformamide (DMF) as modifier and the ionic strength of the buffer solution.

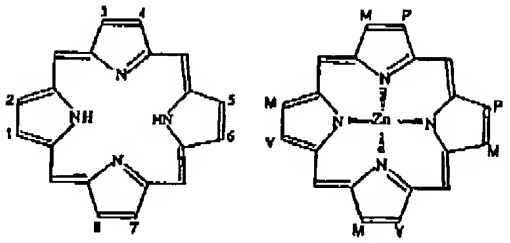
EXPERIMENTAL

Equipment

Separations were performed at ambient temperature using a CE system consisting of a Spellman (Plainview, NY, USA) RHR30 high-voltage power supply, capable of delivering up to 30 kV, and a Shimadzu (Kyoto, Japan) RF535 variable-wavelength fluorescence detector. The excitation and emission wavelengths were set at 405 and 615 nm, respectively. Electropherograms were recorded with a Hewlett-Packard (Palo Alto, CA, USA) Model 3394A integrator. Untreated fused-silica capillary tubing (50 μm I.D.) was obtained from Polymicro Technologies (Phoenix, AZ, USA). A separation tube length of 54.8 cm (effective length 51 cm) was used for all experiments. Samples were injected by hydrostatic siphoning at an elevation of 10 cm for 5 s. The amount injected was estimated to be 1.3 nl.

Chemicals and reagents

The structural formulae of the compounds studied are shown in Fig. 1. Mesoporphyrin IX, deuteroporphyrin IX, pentacarboxylporphyrin I, hexacarboxylporphyrin I, heptacarboxylpor-



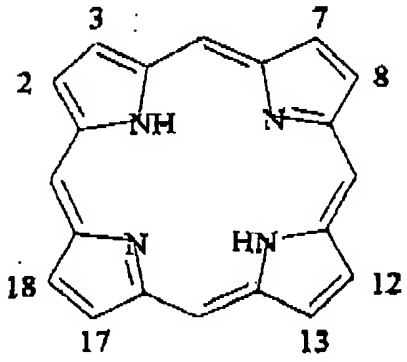
Zinc Protoporphyrin IX (ZP)

Porphyrins	side chain substitution pattern							
	1	2	3	4	5	6	7	8
Deuteroporphyrin IX (DP)	M	H	M	H	M	P	P	M
Mesoporphyrin IX (MP)	M	E	M	E	M	P	P	M
Protoporphyrin IX (PP)	M	V	M	V	M	P	P	M
Coproporphyrin I (CP)	M	P	M	P	M	P	M	P
Pentacarboxylporphyrin I (PeP)	M	P	M	P	A	P	M	P
Hexacarboxylporphyrin I (HxP)	M	P	A	P	A	P	M	P
Heptacarboxylporphyrin I (HP)	A	P	A	P	A	P	M	P
Uroporphyrin I (UP)	A	P	A	P	A	P	A	P

Abbreviation for substitutions:
M = $-CH_3$, P = $-CH_2CH_2COOH$, E = $-CH_2CH_3$, V = $-CH=CH_2$, A = $-CH_2COOH$

Fig. 1. Structures of the porphyrins studied.

Table 1. Structures of porphyrins used in this study.



Porphyrins	Side-chain substitution							
	2	3	7	8	12	13	17	18
Uroporphyrin I	A	P	A	P	A	P	A	P
Heptacarboxylporphyrin I	M	P	A	P	A	P	A	P
Hexacarboxylporphyrin I (cis)	M	P	M	P	A	P	A	P
Hexacarboxylporphyrin I (trans)	M	P	A	P	M	P	A	P
Pentacarboxylporphyrin I	M	P	M	P	M	P	A	P
Coproporphyrin I	M	P	M	P	M	P	M	P

A = $-CH_2COOH$; P = $-CH_3CH_2COOH$; M = $-CH_3$

Various chromatographic techniques have been developed to determine free porphyrin carboxylic acids. Among these methods, high-performance liquid chromatography (HPLC) is a preferred technique due to its efficiency, sensitivity, simplicity and easy quantitation (2,4).

Both normal phase and reversed-phase modes have been tried. In the normal phase mode, an aminopropyl-bonded silica phase was used for the separation of